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Inhibitory effect of *Thymus vulgaris* extract on memory impairment induced by scopolamine in rat

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ABSTRACT

Objective: To investigate the effect of *Thymus vulgaris* (*T. vulgaris*) on learning and memory functions in scopolamine-induced memory deficit in rats. Memory enhancing activity in scopolamine-induced amnesic rats was investigated by assessing the Morris water maze and passive avoidance paradigm.

Methods: A total of 42 male Wistar rats were divided into 6 equal groups as follow: control group: received water, scopolamine treated group: received scopolamine 1 mg/kg for 15 days, two scopolamine + *T. vulgaris* treated groups: received scopolamine and *T. vulgaris* extract 50 and 100 mg/kg body weight per day for 15 days, two intact groups: received *T. vulgaris* extract 50 and 100 mg/kg body weight per day for 15 days.

Results: Administration of *T. vulgaris* extract significantly restored memory and learning impairments induced by scopolamine in the passive avoidance test and Morris water maze test.

Conclusions: *T. vulgaris* extract has repairing effects on memory and behavioral disorders produced by scopolamine and may have beneficial effects in the treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is an age-associated neurodegenerative disorder with many cognitive and neuropsychiatric manifestations that result in progressive disability and eventual incapacitation [1]. Pathological hallmarks of AD lead to loss of forebrain cholinergic neurons and decrease of acetylcholine in the brain of patients with AD appears to be a critical element in producing dementia. The most encouraging treatment for AD is still the using of acetylcholinesterase (AChE) inhibitors [2].

AChE inhibitors include rivastigmine, tacrine, donepezil, and galantamine, while N-methyl-D-aspartate receptor antagonists (memantine) have been also prescribed most recently [1]. The drugs approved for the AD therapy act by counteracting the acetylcholine deficit, that is, they try to enhance the acetylcholine level in the brain [3]. AChE presents some limitations, such as their short half-lives and excessive side effects caused by the activation of peripheral cholinergic systems, which is the most frequent and important side effect of these drug therapies [4].

Scopolamine, a muscarinic cholinergic receptor antagonist, serves as a useful pharmacological tool in producing a model of partial amnesia. Muscarinic acetylcholine receptors mediate the action of acetylcholine [5]. There is considerable evidence that scopolamine causes oxidative stress through the interference with acetylcholine in brain leading to cognitive impairment [6].

Brain oxidative stress was reported following the intra ventricular administration of ethylcholine aziridinium, a toxic

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analog of choline that disrupts high affinity choline transport producing a persistent presynaptic cholinergic hypo function with the induction of amnesia [6]. One factor that plays an important role in the pathogenesis of AD is oxidative stress that is an imbalance between free radicals and antioxidant systems. Oxidative stress is characterized by an imbalance in radical production of reactive oxygen species and antioxidative defense, both are considered to have a major role in the process of age-related neurodegeneration [7].

As well-known, plants have been utilized for medical treatment of various diseases since ages or have been profitable sources for bioactive compounds. Plant extracts may provide a source of new compounds including many drugs that are derived from plant sources. *Thymus vulgaris* (*T. vulgaris*) is one of the best-known medicinal plants of the mint family and grows abundantly in the mountainous areas of the central part of Iran. *T. vulgaris* characterized by the large scale component of free radical scavenger essential oils such as thymol, carvacrol, 8-terpinene, p-cymene and α-pinene. Carvacrol and thymol are the main components of *T. vulgaris* [8]. Active ingredients of *T. vulgaris* are useful in the treatment of convulsions, respiratory diseases, smooth muscle spasm and bloating [9]. Meanwhile, *T. vulgaris* has an antispasmodic action on guinea pig ileum by decreasing the amplitudes of the muscle contractions during peristalsis by affecting the anticholinergic and serotonergic pathways [9].

The hydro-alcoholic extract of *T. vulgaris* can modulate acute and chronic pain in mice [10]. In this study, we investigated whether the *T. vulgaris* extract could inhibit the memory impairment induced by scopolamine through the inhibition of AChE or decrease the oxidative stress. The restored degree of impairment was gauged using both passive avoidance and the Morris water maze tests with or without treatment.

2. Materials and methods

2.1. Preparation of the extract

T. vulgaris plants were purchased from medicinal herbs sellers in Shahrekord and milled finally become a powder. A total of 25 g powder was placed in 70% ethanol for 72 h. The mixture was stirred several times per day. This extract was filtered and dried, then dissolved in water and stored at 4 °C until use [11].

2.2. Measurement of antioxidant activity

Sample stock solutions were diluted to final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/mL in methanol. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) methanol solution was added to sample solutions and allowed to react at room temperature. After 15 min the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity using the following formula [12]:

$$I(\%) = 100 \times \left(A_{control} - A_{sample}\right) / A_{control}$$

2.3. Total phenolic compounds measurement

Total phenolic compounds were determined using a modified version of the Folin–Ciocalteu method. About 0.1 mL of the extract was added to 0.5 mL of Folin–Ciocalteu phenol reagent. The mixture was then allowed to stand for 5 min and 0.4 mL sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. The standard curve was plotted using 12.5, 25.0, 50.0, 62.5, 100.0, and 125.0 mg/L solutions of gallic acid in methanol and water (60:40, v/v). The amount of phenols was obtained based on mg/g in gallic acid equivalent [13].

2.4. Total flavonoid and flavonol determination

Briefly, the amount of total flavonoids in the *T. vulgaris* extract was determined using the colorimetric method. Solution with 1.5 mL of methanol (60%), 1 mL of 2% aluminum chloride, and 6 mL of 5% potassium acetate was added to 1 mL of the *T. vulgaris* extract. The mixture was left at room temperature for 40 min. The absorbance of the reaction mixture was then measured at 415 nm. The aluminum chloride colorimetric method was employed for flavonol determination, but the incubation period was 150 min and the absorbance of the reaction mixture was determined at 440 nm. Total flavonoids and flavonols were expressed in terms of rutin equivalent (mg/g), which is a common reference compound [14].

2.5. Animals

Male Wistar rats, weighing 150–250 g, were obtained from Pasteur Institute (Tehran, Iran). Rats were housed in cage at 25 °C with a controlled 12 h light–dark cycle. Food and water were freely available. All experiments were executed in accordance with the Guide for the Care and Use at Laboratory Animals and were approved by Research and Ethics Committee of Medical Sciences School of Shahrekord University.

Animals were randomly divided into 6 groups with 7 rats in each group: Control group, received distilled water for 15 days via *i.p.* injection; Scopolamine (SCOP) treated group: rats received scopolamine at a dose of 1 mg/kg for 15 days via *i.p.* injection; Scopolamine + *T. vulgaris* (SCOP-TV) 50, 100 groups: rats received *T. vulgaris* extract at 50 and 100 mg simultaneously with scopolamine at 1 mg/kg for 15 days; Intact groups received only *T. vulgaris* extract at 50 and 100 mg/kg respectively, for 15 days.

Following the behavioral tests, under deep anesthesia the blood sample collected from heart and the brains was quickly removed. Different parts of the brains such as hippocampus, cortex and subcortex were separated.

2.6. Water maze test

The water maze test is also a widely accepted method for memory test. A circular water pool was filled with milky water kept at 22–25 °C. An escape platform was submerged below the surface of the water in position. On training trials, the rats were placed in a pool of water and allowed to remain on the platform for 10 s and were then returned to the home cage during the second-trial interval. The rats that did not find the platform within 60 s were placed on the platform for 10 s at the end of trial. Animals were given 4 trials daily for 4 consecutive days. On the 15th day, rats were individually subjected to a probe trial session by removing the platform and were allowed to swim for 120 s to search for the platform.

2.7. Passive avoidance test

The passive avoidance test is widely accepted as a simple and rapid method for memory test. The passive avoidance response was determined using a step-through apparatus that consisted of a lighted compartment and a dark compartment with a grid floor. The 2 compartments were separated by a guillotine door and each had a grid floor through which a foot shock could be delivered. This test was performed for each rat during the 4 days. On the 1st and 2nd days of testing, each rat was placed on the apparatus and left for 5 min to habituate to the apparatus. On the 3rd day, an acquisition trial was performed. On the training day, the rat was placed in the lighted compartment, facing away from the dark compartment and allowed to explore for 20 s. After 20 s the guillotine door was lifted. When the rat entered the dark compartment the guillotine door was closed, and the latency to enter was recorded. After the door was closed, a foot shock (1 mA, 1 s duration) was delivered through the stainless steel rods. On the 4th day (24 h after training), the rat was returned to the lighted compartment. After 5 s, the guillotine door was lifted. When the rat entered the dark compartment, the guillotine door was closed, and the step-through latency for animals was recorded.

2.8. Assessment of antioxidant power in brain and serum

Antioxidant power of brain or serum was determined by measuring its ability to reduce Fe^{3+} to Fe^{2+} established as the ferric reducing-antioxidant power (FRAP) test. The reagents included 300 mmol/L acetate buffer solution (pH 3.6) with 16 mL acetic acid per 1 L of buffer solution, 10 mL trypyridyl-s-triazine in 40 mmol/L HCl and 20 mmol/L $FeCl_3$. A total of 50 μL of supernatant or serum was added to 1.5 mL of freshly prepared reagent warmed at 37 °C. After 10 min, the complex between Fe_2^+ and trypyridyl-s-triazine gives a blue color with absorbance at 593 nm. Data were expressed as μmol/L per g of wet tissue or μmol/L per L serum [15].

2.9. Measurement of plasma malondialdehyde (MDA)

Plasma MDA levels were estimated by high performance liquid chromatography. A C18 column was used for separation of the thiobarbituric acid-MDA adduct. Briefly, to 100 μL plasma or standard, 100 μL sodium dodecyl sulfate (8.1%) and 2.5 mL thiobarbituric acid (TBA)/buffer (prepared by dissolving of 0.53% thiobarbituric acid in 20% acetic acid as adjusted to pH 3.5 with NaOH) were added. The tubes were covered with caps and incubated at 95 °C for 60 min. The reaction was stopped by placing tubes on ice followed by centrifugation at 4 000 r/min for 10 min to separate 2 phases. The supernatant (20 μL) was injected into the high performance liquid chromatography system.

2.10. MDA levels of brain

For assessment of lipid peroxidation in brain tissue, after sacrifice, the animals were decapitated and the brains rapidly removed. Brain tissues were homogenized in ice-cold Tris-HCl buffer (50 mmol/L, pH 7.4) for 2 min at 5 000 r/min. The homogenized solution was then centrifuged for 60 min at 4 300 r/min. The optimized TBA assay was as follows: 100 μL supernatant was treated with 10 μL butylated hydroxytoluene 0.5 mol/L, followed by addition of 200 μL 8.1% sodium didosylesolphate and 1.5 mL TBA. Then samples were heated for 60 min in boiling water bath (at 95 °C). After cooling at 4 °C, 3 mL butanol-pyridine was added to the samples and centrifuged at 3 900 r/min for 10 min. Finally, an aliquot (120 μL) was directly injected onto the high performance liquid chromatography system [16].

2.11. Plasma nitric oxide (NO) levels

Serum samples were stored at −20 °C and deproteinized by adding $ZnSO_4$ and NaOH solutions. After centrifuging, the supernatant was recovered and diluted by glycine buffer. Cadmium granules were rinsed 3 times with deionised distilled water and swirled in a 5 mmol/L $CuSO_4$ solution in glycine-NaOH buffer for 5 min to activate. Freshly activated cadmium granules were added to pretreated deproteinized serum. After continuous stirring for 10 min, the samples were transferred to appropriately labeled tubes for nitrite determination by Griess reaction. Griess reagent 1 (1% sulfanilamide in 5% phosphoric acid) was added to the sample tubes, incubated for 10 min at room temperature and protected from light. Finally, Griess reagent 2 was dispensed (0.1% N-naphthylethylenediamine dihydrochloride in water) to all samples and absorbance was measured within 10 min in a spectrophotometer with a 540 nm filter [17].

2.12. Statistical analysis

Data were expressed as mean ± SEM and processed by commercially available software SPSS 11.0. All results were compared using One-way ANOVA and *post-hoc* Tukey test. *P*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Standardization of T. vulgaris extract

Total amount of phenolic compounds in *T. vulgaris* extract was 209 mg gallic acid equivalent per 1 g dried extract. Total amount of flavonoid and flavonol compounds were 21.8 mg and 10.7 mg, respectively per 1 g of dry matter.

3.2. Effect of T. vulgaris extract on DPPH free radical scavenging

The antioxidant activity of *T. vulgaris* extract was assessed on the basis of radical scavenging of the stable DPPH free radical. *T. vulgaris* extract showed strong free radical scavenging activity against DPPH radicals, with an IC_{50} value of 70 μg/mL (Table 1).

3.3. Passive avoidance response

The latency time to enter the dark chamber in the acquisition trial was significantly longer in the rats given scopolamine, as compared to the control group. Initial latency (T1) significantly reduced in Intact + 100 TV group when compared with SCOP group ($P < 0.05$). The step-through latency time (T2) in the SCOP group in the retention trial was significantly shorter than

Table 1
DPPH radical scavenging activity of *T. vulgaris* extract at different concentrations.

Concentration (μg/mL)	Inhibition (%)
10	16.89
20	19.80
30	25.80
40	33.00
50	41.74
60	54.10
70	59.60*
80	65.40
90	70.80
100	80.30

*: IC_{50} value (%).

in the control group ($P < 0.05$). Furthermore, the step through latency (T2) time of intact rats treated with *T. vulgaris* extract at dose of 100 mg/kg significantly increased compared to SCOP rats (Figure 1).

3.4. Morris water maze swimming test

The time spent by the animal, searching for the missing platform in target quadrant with respect to other quadrant on day 5 was noted as an index of retrieval memory. SCOP group significantly spent less time in the correct quadrant compared with control group in the probe trail ($P < 0.05$). In the probe trial following the last training session *T. vulgaris* with doses of 100 mg/kg increased the swimming time in the target quadrant in intact group (Figure 2).

3.5. Plasma antioxidant level

Scopolamine treatment significantly decreased the plasma antioxidant level compared to the control group ($P < 0.05$). *T. vulgaris* extract with doses of 50 and 100 mg/kg in intact groups significantly increased plasma antioxidant levels compared with SCOP group ($P < 0.05$). Plasma antioxidant levels in SCOP+50TV group significantly higher than SCOP group (Figure 3).

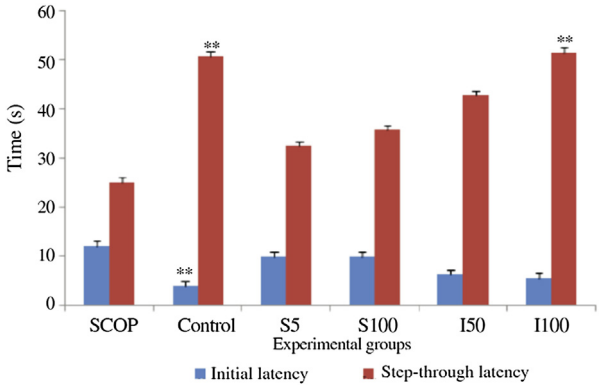

Figure 1. The initial latency and step-through latency in the passive avoidance response. :
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; *: $P < 0.05$; **: $P < 0.01$; SCOP *vs.* control, SCOP + 50TV, SCOP + 100TV, Intact + 50TV, Intact + 100TV groups ($n = 7$).

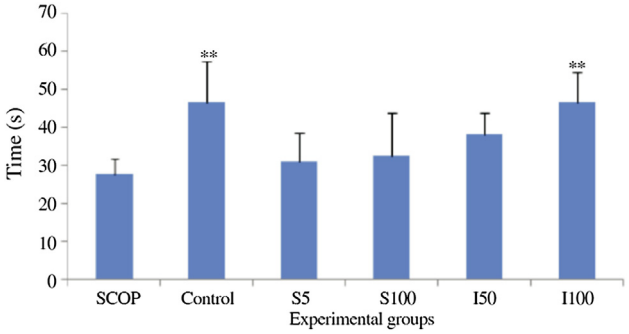

Figure 2. The time spent in target quadrant during the probe trial.
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; **: $P < 0.01$.

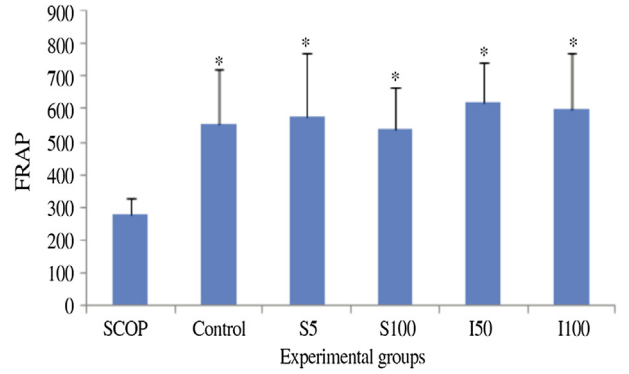

Figure 3. Plasma antioxidant levels in experimental groups.
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; *: $P < 0.05$.

3.6. Brain antioxidant levels

T. vulgaris extract significantly increased antioxidant levels of hippocampus, cortex and subcortex in SCOP+50TV, SCOP+100TV groups compared with SCOP group. *T. vulgaris* extract at dose of 50 mg/kg in intact groups increased antioxidant levels of cortex compared with SCOP group (Figure 4).

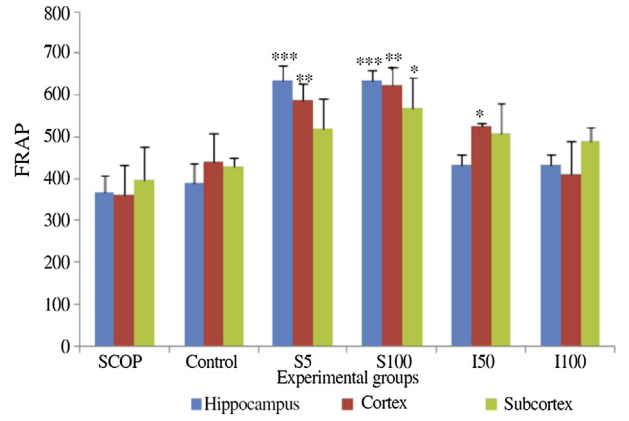

Figure 4. Brain antioxidant levels (FRAP) in experimental groups.
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

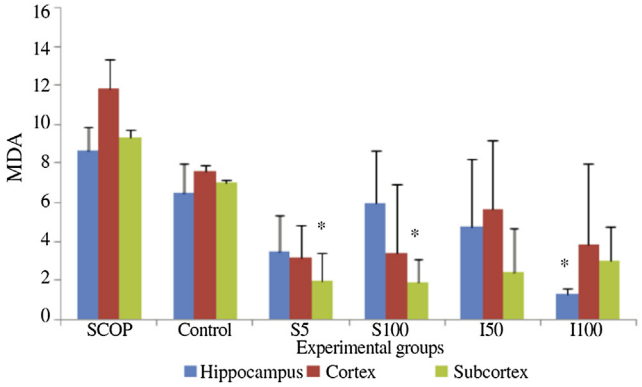

Figure 5. Brain MDA levels in experimental groups.
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; $^{*}P < 0.05$.

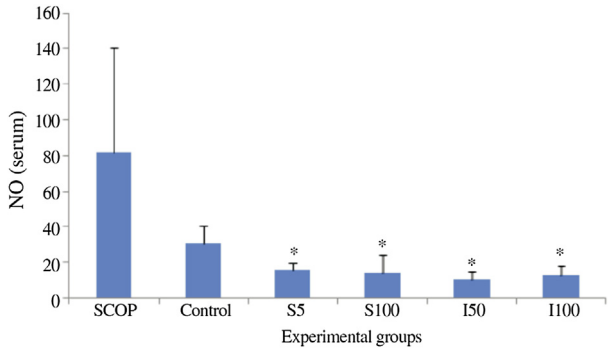

Figure 6. Plasma NO levels in experimental groups.
S5: SCOP + 50TV; S100: SCOP + 100TV group; I50: Intact + 50TV; I100: Intact + 100TV; $^{*}P < 0.05$.

3.7. Brain MDA level

Scopolamine increased MDA levels of hippocampus, cortex and subcortex in SCOP group compared with control group but these changes not statistical significant. *T. vulgaris* extract with dose of 50 and 100 mg/kg significantly decreased subcortex MDA levels in SCOP+50TV and SCOP+100TV groups. *T. vulgaris* extract with dose of 100 mg/kg in intact group significantly decreased hippocampus MDA levels compared with SCOP group (Figure 5).

3.8. Plasma NO levels

Scopolamine treatment nonsignificantly increased plasma NO levels when compared with control group. *T. vulgaris* extract significantly decreased plasma NO levels in SCOP+50TV, SCOP+100TV, Intact+50TV, Intact+100TV groups compared with SCOP group (Figure 6).

4. Discussion

AD is a chronic neurodegenerative disease that usually starts slowly and gets worse over time, and it is the cause of more than 50% of dementia patients developing in the population older than 60 years of age [18]. Brain aging is known to be related to decrease in acetylcholine level, neuronal loss, increased inflammation, and oxidative stress [19]. In AD patients acetylcholine levels depleted in the brain. Centrally acting cholinergic drugs have been reported to increase the regional cerebral blood flow of acetylcholine in the brain regions [20]. The maintaining of the acetylcholine level in the brain is important for the cure of AD patients. One method for this is blocking the activity of AChE, the enzyme-degrading acetylcholine [20].

The anticholinergic drugs, like scopolamine, can disrupt short-term or working memory in humans and animals by blocking muscarinic receptors in these brain regions [21]. The AChE inhibitors, which enhance the availability of acetylcholine in the synaptic cleft, were able to reverse the scopolamine induced deficit [21]. The novel AChE inhibitors from plant sources could be valuable alternatives in the context of the treatment of AD. Several studies have showed the cognition-enhancing properties of natural products and their components using different animal models [12, 22, 23].

Thymohydroquinone exhibited the strongest AChE inhibitory effect over the range of concentrations. The AChE inhibitory effect exerted by carvacrol was 10 times stronger than that exerted by its isomer thymol, although thymol and carvacrol have a very similar structure [24]. *T. vulgaris* indicates neuroprotective effects due to their small molecular size and lipophilicity, volatile constituents and liberated volatile aglycones from glycosides are likely to readily cross the blood–brain barrier [25]. The previous study indicated that, after galantamine, thymohydroquinone exhibited the strongest AChE inhibitory effect [24].

Scopolamine also causes oxidative stress through the interference with acetylcholine in brain leading to cognitive impairment [6]. Scopolamine significantly increases AChE and MDA levels in the cortex and hippocampus [6]. This study aimed at investigating whether such impaired cognition due to scopolamine administration is associated with altered oxidative stress indices. The cognitive-enhancing activity of *T. vulgaris* extract on the scopolamine induced memory impairments in rat was investigated using passive avoidance test, Morris water maze test and biochemical assessments.

The passive avoidance task is one of the most frequently employed methods for evaluating memory enhancing effects, *in vivo*. *T. vulgaris* extract significantly reversed the shorter step-through latency induced by scopolamine. In probe trail session in Morris Water Maze test the scopolamine-induced reduction in swimming times within the platform quadrant was significantly ameliorated by *T. vulgaris* extract. The *T. vulgaris* compounds such as thymol, carvacrol and different quinines have anti-inflammatory effects *in vitro* [26]. The strong AChE inhibitory effect was observed with the compounds that have been previously identified as strong antioxidants [27].

Our results indicated that scopolamine increased brain MDA levels. *T. vulgaris* extract decreased brain MDA levels in experimental groups. MDA is one of the major aldehydes formed after breakdown of lipid hydroperoxides. Therefore, it is considered as a good biomarker of the involvement of free radical damage in pathologies associated with oxidative stress [28]. The brain is considered to be more susceptible to peroxidative damage than other tissues due to the high content of their polyunsaturated lipid-rich neural parenchyma, high oxygen utilization and low antioxidative enzymes. Furthermore, previous studies indicated that oxidative stress is one of the earliest events in pathogenesis of memory impairment [29]. Oxidative stress was produced by free radicals, *i.e.*, reactive oxygen species generated by oxygen and nitrogen-based molecules that have unpaired electrons [29].

NO is a weak free radical formed during the conversion of L-arginine to l-citrulline by NO synthase. NO when combined with superoxide generates a potent radical, peroxynitrite, which activates lipid peroxidation [30]. Our results showed that scopolamine significantly increased serum NO levels. *T. vulgaris* extract significantly decreased serum NO levels in experimental groups. The other studies confirmed our studies, thymol and carvacrol alleviated cognitive impairments caused by increased Aβ levels in rats [31].

Our results suggested that the anti-amnesic effect of *T. vulgaris* extract on scopolamine-induced memory impairment may be related to the antioxidant activity of extract or mediation of the cholinergic nervous system [32–34]. Therefore, other plants which have antioxidant activity [35–39] might reduce amnesia.

Conflict of interest statement

We declare that we have no conflict of interest.

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